Selective loss of the uncoupling protein from light versus heavy mitochondria of brown adipocytes after a decrease in noradrenergic stimulation *in vivo* and *in vitro*

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The relative stability against a decrease in adrenergic stimulation of the uncoupling protein (UCP) incorporated into different mitochondrial fractions was investigated in brown-fat-cell cultures. Cultures were initiated with undifferentiated cells from young mice and were acutely stimulated with noradrenaline at confluence (day 7). Cells were harvested just after the finish of the 24 h stimulation treatment or 24 h later, and three mitochondrial fractions were isolated by differential centrifugation: the M1 fraction (1000 *g*), the M3 fraction (3000 *g*) and the M15 fraction (15000 *g*). The results obtained *in vitro* indicate that removal of adrenergic stimulation determines a selective loss of UCP from the lightest mitochondrial fractions (M3 and M15). Similar results were obtained in a situation *in vivo* (24 h starvation in mice) which is known to lead to a decreased noradrenaline input to brown adipose tissue, with decreased UCP levels. Thus brown adipocytes possess different mitochondrial subpopulations, which exhibit characteristic changes in their UCP turnover in response to thermogenic signals.

INTRODUCTION

The uncoupling protein (UCP) or thermogenin is a brown-fat-specific mitochondrial-inner-membrane protein which is the rate-limiting factor for thermogenesis in brown adipose tissue (BAT) [1–4].

UCP gene expression has been shown to be under noradrenergic control both *in vivo* and *in vitro*. BAT recruitment and increased UCP expression are seen under physiological conditions which are associated with an increased sympathetic stimulation of BAT with enhanced noradrenaline (NA) release in the tissue, such as cold exposure or chronic overfeeding [5–8], whereas BAT atrophy and decreased UCP expression are seen in various situations associated with a significant decrease in sympathetic activity to BAT, such as fasting [9–12] and return of cold-acclimated rodents to thermoneutral environments [13]. Similarly, in brown-fat cells differentiating *in vitro*, NA has been proved competent in stimulating both UCP mRNA synthesis and translation to mature UCP [14–16]. The presence of NA in those cultures is essential not only to increase the level of UCP, but also to maintain this elevated level, since the specific UCP content falls as soon as NA is removed [17].

The mechanisms underlying the decrease in the UCP levels during BAT atrophy seem to include both down-regulation of UCP synthesis and active degradation of pre-existing UCP molecules. It has been shown that a marked loss of UCP mRNA precedes the loss of UCP from mitochondria during reacclimation of cold-exposed animals to warmth [18,19], fasting [20], and after the cessation of adrenergic stimulation of brown adipocytes cultured *in vitro* [21]. On the other hand, the existence of a degradative system specifically directed towards UCP was suggested by results showing that, in cultured brown adipocytes transiently adrenergically stimulated, the induced UCP was rapidly and specifically degraded after removal of NA, this degradation being dependent on uninhibited protein synthesis [17]. A selective proteolytic system was also claimed to be involved in the decrease in the specific BAT UCP content during fasting in mice [22,23]. Interestingly, an impaired starvation-induced loss of mitochondrial protein and of UCP has been described in BAT of rats with diet-induced obesity [24,25], suggesting a failure of the proteolytic pathways to respond to fasting in these animals.

All the above studies were based on the assumption that only one type of mitochondria is present in the cell, endowed with homogeneous metabolic features. However, it has been shown that mitochondria from both liver and BAT may be fractionated into different subpopulations, which exhibit remarkable differences not only in their dimensions, but also in their respiratory features and their response to cold exposure [26,27]. The aim of this work was to investigate the relative stability against a decrease in noradrenergic stimulation of the UCP incorporated into different mitochondrial fractions and its possible physiological significance. To address the question, we have used a model *in vitro* of BAT atrophy consisting of brown-fat-cell cultures transiently adrenergically stimulated, and compared the results *in vitro* with those obtained in a well-known model *in vivo* of BAT atrophy, such as starvation. Thus, the distribution of UCP among mitochondrial fractions was analysed in cultured cells fractionated just after having received the NA treatment or 24 h later, and in control and 24 h-starved mice.

MATERIALS AND METHODS

Animals

The animals were NMRI male mice from Letica aged 3 weeks on arrival. They were housed in group cages (10 mice/cage) at 23 °C, with a 12 h-light/12 h-dark cycle (lights on at 08:00 h) and free access to tap water and pelleted standard diet. The mice were left for 1 week under these conditions before being taken for experiments. For starvation experiments, 4-week-old mice ac-

Abbreviations used: UCP, uncoupling protein, or thermogenin; BAT, brown adipose tissue; NA, noradrenaline; COX, cytochrome *c* oxidase.

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climated at 23 °C were housed in pairs; some pairs were kept as fed controls and the remainder were starved for 24 h. Food was removed at between 09:00 h and 10:00 h. Animals were killed by cervical dislocation.

Experiments *in vivo*

Interscapular, axillar and cervical BAT depots from two mice were combined and homogenized in Hepes/sucrose buffer (0.25 M sucrose/2 mM Hepes/0.2 mM EDTA, pH 7.4; final concn. 15%, w/v) with 10 strokes of a Teflon/glass power-operated homogenizer. The homogenate was filtered through two layers of gauze and centrifuged at 15000 *g* for 15 min. The hard-packed fat layer and supernatant were discarded, and the pellet, consisting of cell debris, nuclei and mitochondria, was resuspended in the original volume of the same buffer and used to isolate the different mitochondrial subpopulations (see below).

Experiments *in vitro*

BAT precursor cells from 4-week-old mice were prepared, inoculated and cultured as described previously [17], except that 5 ml culture flasks were used. Routinely, 20 flasks were inoculated with a cell suspension prepared from 20 mice. A 0.5 ml portion of pooled final cell suspension was inoculated per flask and 4.5 ml of culture medium added. The medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn-calf serum, 4 nM insulin, 10 mM Hepes and 126 μM sodium ascorbate, and with 50 units of penicillin and 50 μg of streptomycin per ml. The cells were grown at 37 °C in an atmosphere of 8% CO_2 in air. The culture medium was changed on days 1 and 3 after inoculation. At confluence (day 7), the cells were treated with two doses of 10 μM (final concn.) NA added at 0 h and 8 h. On day 8, immediately after finishing the 24 h NA treatment, cells from half of the flasks were harvested (sample NA^+), and NA was removed from the remaining cells, which were rinsed with 5 ml of DMEM before receiving fresh medium and kept in culture. These cells were harvested on day 9, 24 h after removal of NA (sample NA^-). For harvesting, the cells were rinsed twice with ice-cold PBS (137 mM NaCl/2.7 mM KCl/10 mM phosphate buffer, pH 7.4) and then scraped into 2.5 ml of PBS and transferred to a centrifuge tube. Cells from 10 flasks were pooled, precipitated by centrifugation at 500 *g* for 10 min, resuspended in 1 ml of Hepes/sucrose buffer and homogenized by 40 strokes in a small well-fitting glass/glass Dounce homogenizer (2 ml size), followed by four passes through a hypodermic needle.

Isolation of mitochondria

Nuclei and cell debris were removed from BAT homogenates and homogenized cultured cells by centrifugation at 500 *g* for 10 min at 4 °C. The pellet was washed and the resulting supernatant was subjected to three sequential centrifugation steps, each lasting 10 min, at 1000 *g*, 3000 *g* and 15000 *g*. The pellets, called M1, M3 and M15 respectively, were resuspended in a minimal volume of Hepes/sucrose buffer.

Parameters determined

Protein concentration, cytochrome *c* oxidase (COX) activity and UCP content were routinely determined in samples of BAT homogenates or homogenized cultured cells and in each mitochondrial fraction. Protein concentration was measured by the method of Bradford [28], with BSA as standard. COX activity was measured at 37 °C by a spectrophotometric method that monitors changes in absorbance during the oxidation of reduced ferrocytochrome [29]. UCP was measured by immunoblotting as previously described [17]; for quantitative analysis, the bands were scanned with a Bio-Image computing densitometer (Millipore).

Specific GDP binding to BAT mitochondrial subpopulations of control and starved mice was measured as described previously [25], with slight modifications. The final mitochondrial pellets were resuspended in Hepes/sucrose buffer at a final concentration of 5–10 mg of protein/ml. Mitochondrial pellets (0.2 mg of protein) were incubated at 30 °C in 2 ml of buffer containing 100 mM [U-^{14}C]sucrose (6 kBq/ml), 10 μM [8-^{3}H]GDP (ammonium salt) (10 kBq/ml), 20 mM Tes, 1 mM EDTA and 5 μM rotenone. The concentration of GDP used was previously shown to give the maximum GDP binding assayed with 0.3 mg of BAT mitochondrial protein from fed and starved animals [24]. Non-specific binding was assessed by addition of 25 μl of 10 mM unlabelled GDP. [U-^{14}C]Sucrose was used to assay the intramitochondrial space. Specific and non-specific GDP binding was performed by filtration through 0.45 μm-pore filters (Sartorius).

Characterization of mitochondrial fractions was performed by determining, in addition to UCP levels and COX activity values, the activity of several marker enzymes for various cellular compartments. Lactate dehydrogenase and catalase were determined as described in [30] and [30a]; 5′-nucleotidase and acid phosphatase were determined with kits from Sigma.

RESULTS

Enzymic profile of mitochondrial fractions

Determination of marker enzyme activities showed that fractions M1, M3 and M15 obtained from BAT homogenates of fed mice were clearly enriched in mitochondria (Table 1). Minor contamination by other cellular organelles, such as peroxisomes and lysosomes, as well as by plasma-membrane fragments, was apparent in all three fractions, and especially in the M15 fraction.

Experiments *in vitro*

About 22% of total protein and 65% of total COX activity present in the cell lysates were recovered among the three mitochondrial fractions collected (M1 + M3 + M15), in both NA^+ (harvested on day 8, just after finishing the 24 h NA treatment) and NA^- (harvested on day 9, 24 h after removal of NA) samples. Protein content of cell lysates increased from day 8 of culture to day 9 by approx. 20% in spite of NA removal (from 237 ± 67 to 284 ± 79 μg/flask; $n = 5$), whereas the corresponding total COX activity values remained unchanged (from 25.5 ± 8.9

Table 1 Enzymic profile of M1, M3 and M15 mitochondrial fractions

BAT homogenates of fed mice were fractionated, and the marker-enzyme activities shown below were measured in mitochondrial fractions, after sonication, as described in the Materials and methods section. Data are expressed relative to the corresponding specific activity in crude homogenate, and correspond to means ± S.E.M. of 3–4 animals.

	M1	M3	M15
COX	9.14 ± 1.54	8.26 ± 2.48	10.2 ± 3.71
Catalase	1.56 ± 0.46	2.22 ± 0.55	2.70 ± 0.42
5′-Nucleotidase	1.27 ± 0.33	1.66 ± 0.17	2.05 ± 0.71
Acid phosphatase	1.13 ± 0.09	1.67 ± 0.15	1.54 ± 0.17
Lactate dehydrogenase	0.14 ± 0.03	0.14 ± 0.04	0.18 ± 0.06

Table 2 Distribution of protein, COX activity and UCP among mitochondrial fractions of cultured brown-fat cells after a 24 h NA treatment (NA^+) and 24 h after removal of NA (NA^-)

Cells were treated with two doses of 10 μM NA on day 7 after inoculation and were harvested either immediately after the 24 h NA treatment (samples NA^+) or 24 h after removal of NA (samples NA^-). Mitochondrial fractions were obtained by differential centrifugation at 1000 *g* (M1), 3000 *g* (M3) and 15000 *g* (M15). Data are expressed as percentage of total protein, total units of COX activity and total UCP recovered among the three mitochondrial fractions, and correspond to means ± S.E.M. of 5 independent cultures. NA^+ and NA^- data were analysed by a two-way ANOVA; contrasts between means were assessed by the Newman–Keuls test. The ratio NA^-/NA^+ was calculated for each individual culture. Significant differences: $^*P < 0.05$ M3 or M15 versus M1; N.S., not significant ($P > 0.05$).

	NA^+	NA^-	NA^-/NA^+	ANOVA
(a) Protein (%)				
M1	40.4 ± 8.8	43.0 ± 10.8	1.05 ± 0.12	Fraction: N.S.
M3	30.9 ± 4.8	34.3 ± 4.9	1.18 ± 0.19	NA removal: N.S.
M15	28.7 ± 5.8	22.8 ± 6.1	0.763 ± 0.094	Interaction: N.S.
(b) COX activity (%)				
M1	39.5 ± 8.1	42.5 ± 14.2	0.975 ± 0.143	Fraction: $P < 0.05$
M3	40.4 ± 3.9	38.1 ± 8.5	0.935 ± 0.202	NA removal: N.S.
M15	20.1 ± 4.8	19.4 ± 7.6	0.826 ± 0.280	Interaction: N.S.
(c) UCP (%)				
M1	59.6 ± 10.0	73.8 ± 8.9	1.33 ± 0.14	Fraction: $P < 0.05$
M3	28.2 ± 5.1*	20.3 ± 6.1*	0.674 ± 0.099*	NA removal: N.S.
M15	12.2 ± 4.9*	5.98 ± 3.02*	0.413 ± 0.134*	Interaction: N.S.

Table 3 UCP content, COX activity values and UCP/COX ratios of mitochondrial fractions of cultured brown-fat cells after a 24 h NA treatment (NA^+) and 24 h after removal of NA (NA^-)

Cultures were handled and mitochondrial fractions obtained as described in the Materials and methods section. Units of COX activity are given as nmol of ferrocytochrome oxidized/min; UCP is given as arbitrary units for bands in immunoblots. Data correspond to means ± S.E.M. of 5 independent cultures. Na^+ and NA^- data were analysed by a two-way ANOVA; contrast between means were assessed by the Newman–Keuls test. The ratio NA^-/NA^+ was calculated for each individual culture. Significant differences; $^*P < 0.05$ NA^+ versus NA^-; †$P < 0.05$ M3 or M15 versus M1; ‡$P < 0.05$ M15 versus M3; N.S., not significant.

	NA^+	NA^-	NA^-/NA^+	ANOVA
(a) Total UCP content (units/fraction)				
M1	4.20 ± 0.80	3.42 ± 0.71	0.950 ± 0.230	Fraction: $P < 0.05$
M3	2.40 ± 0.98	1.49 ± 0.88	0.493 ± 0.119	NA removal: N.S.
M15	1.21 ± 0.76	0.480 ± 0.358†	0.349 ± 0.153	Interaction: N.S.
(b) Specific UCP content (units/mg of protein)				
M1	25.4 ± 2.6	18.9 ± 2.3	0.779 ± 0.112	Fraction: $P < 0.05$
M3	14.8 ± 1.5†	5.25 ± 1.01*†	0.360 ± 0.067†	NA removal: $P < 0.05$
M15	6.50 ± 1.47†‡	1.92 ± 0.63*†	0.349 ± 0.160	Interaction: N.S.
(c) Total COX activity (units/fraction)				
M1	56.6 ± 21.1	39.4 ± 8.6	1.04 ± 0.39	Fraction: N.S.
M3	46.2 ± 5.3	48.0 ± 19.4	1.29 ± 0.67	NA removal: N.S.
M15	20.7 ± 3.2	22.2 ± 8.2	1.00 ± 0.37	Interaction: N.S.
(d) Specific COX activity (units/mg of protein)				
M1	304 ± 75	224 ± 52	0.817 ± 0.171	Fraction: N.S.
M3	463 ± 145	234 ± 64	0.810 ± 0.362	NA removal: N.S.
M15	200 ± 54	174 ± 89	0.879 ± 0.273	Interaction: N.S.
(e) UCP/COX ratio (arbitrary units)				
M1	10.7 ± 2.7	11.2 ± 3.1	1.12 ± 0.29	Fraction: $P < 0.05$
M3	6.41 ± 3.47	2.53 ± 0.45†	0.661 ± 0.148	NA removal: N.S.
M15	5.81 ± 3.22	1.84 ± 0.79†	0.385 ± 0.069	Interaction: N.S.

to 24.0 ± 5.7 nmol of substrate oxidized/min per flask; $n = 5$). The increase in protein content is in agreement with previous observations showing a NA-independent increase of total protein content of cultured brown-fat cells after apparent confluence [17,31]. The distribution of protein and COX activity among mitochondrial fractions is shown in Tables 2(a) and 2(b): no significant differences between fractions were found, but a certain gradation was observed, with more protein and COX activity recovered in the heaviest fraction (M1) than in the lightest fraction (M15). This pattern did not change upon removal of NA, the corresponding NA^-/NA^+ ratios being close to 1 in all cases.

As expected [14,17], treatment of confluent brown adipocytes with NA induced UCP appearance compared with control non-treated cultures, which exhibited undetectable or very low basal levels of UCP (results not shown), and removal of NA was accompanied by a decrease in the total UCP content of cells (31% on average, although wide differences were found among individual cultures). Most of the UCP was recovered in the M1 fraction, in both NA^+ and NA^- samples. Upon removal of NA, the percentage of UCP recovered in the M1 fraction increased, while the percentage recovered in fractions M3 and M15 decreased (Table 2c), already suggesting a selective loss of UCP from medium and light mitochondria. This is better seen when total and specific UCP contents of mitochondrial fractions are considered and compared with a general mitochondrial parameter, such as COX activity (Table 3). Total UCP content of fractions M3 and M15 decreased more markedly upon removal of the NA stimulus than that of fraction M1 (Table 3a). The specific UCP content followed a similar pattern, with significant decreases in fractions M3 and M15, but not in fraction M1 (Table 3b). Furthermore, a decrease in the specific UCP content of fractions M3 and M15 occurred in the absence of significant changes in the corresponding COX specific-activity values (Table 3d). From UCP levels and COX activity values, the ratio between both parameters was calculated, to evaluate further whether the changes observed were specific for the thermogenic parameter, as compared with general mitochondrial parameters (Table 3e); the UCP/COX ratio remained practically unchanged in fraction M1 and tended to decrease in fractions M3 and, more markedly, in M15, indicating a selective loss of UCP from the lightest mitochondria on cessation of adrenergic stimulation.

Experiments *in vivo*

In order to evaluate further the findings concerning UCP degradation in cell cultures, their relationship to similar processes *in vivo* was studied. The model used *in vivo* consisted of mice which were starved for 24 h, a treatment which is known to lead to a decrease in NA input into BAT and to decreased UCP levels [9–12].

As expected, 24 h starvation in mice resulted in losses of total body weight (13.6%), total BAT weight (39%) and total (M1 + M3 + M15) BAT UCP content (18%). However, we could not detect a significant decrease in BAT mitochondrial mass under the starvation conditions used, in contrast with previous reports [11,22,32], and thus total protein and total and specific COX activity values remained practically unchanged upon starvation in all mitochondrial fractions (Table 4). This apparent discrepancy could be due to the fact that longer starvation periods (36–48 h) were used in the previous reports (see the Discussion section, below).

Different changes in the thermogenic parameters in response to 24 h starvation were observed among the mitochondrial fractions considered. Total UCP content decreased in fraction M15, remained unchanged in fraction M3 and tended to increase in fraction M1; the specific UCP content followed the same

Table 4 Effect of 24 h starvation on body weight, BAT weight, protein and COX activity from different BAT mitochondrial fractions

Units of COX activity are given as μmol of ferrocytochrome oxidized/min. Data correspond to means ± S.E.M. of 7 determinations (2 animals each), except for body-weight and BAT-weight data, which are the averages of all 14 animals. The starved/fed ratio was calculated from the corresponding mean values. Protein and COX data were analysed by a two-way ANOVA; contrasts between means were assessed by the Newman–Keuls test. Body- and BAT-weight data were assessed by Student's t test. Significant differences: $^*P < 0.05$ fed versus starved; N.S., not significant.

	Fed	Starved	Starved/fed ratio	ANOVA
Body weight (g)	24.3 ± 0.7	21.0 ± 0.7*	0.864	
BAT weight (mg)	194 ± 12	118 ± 6*	0.608	
Total protein recovered (μg/tissue)				
M1	644 ± 66	697 ± 144	1.082	Fraction: N.S.
M3	671 ± 117	671 ± 108	1.000	Starvation: N.S.
M15	839 ± 118	621 ± 43	0.740	Interaction: N.S.
Specific COX activity (units/mg of protein)				
M1	1.32 ± 0.16	1.69 ± 0.14	1.280	Fraction: N.S.
M3	1.42 ± 0.15	1.70 ± 0.13	1.197	Starvation: $P < 0.05$
M15	1.47 ± 0.16	1.75 ± 0.11	1.190	Interaction: N.S.
Total COX activity (units/tissue)				
M1	0.81 ± 0.09	1.13 ± 0.19	1.400	Fraction: N.S.
M3	0.89 ± 0.11	1.10 ± 0.14	1.240	Starvation: N.S.
M15	1.21 ± 0.17	1.08 ± 0.08	0.893	Interaction: N.S.

Table 5 Thermogenic parameters of BAT mitochondrial fractions from fed and 24 h starved mice

UCP is given as arbitrary units for bands in immunoblots. Data correspond to means ± S.E.M. of 7 determinations (2 animals each). Statistical differences were analysed by a two-way ANOVA; contrasts between means were assessed by the Newman–Keuls test. The starved/fed ratio was calculated from the corresponding mean values. Significant differences: $^*P < 0.05$ fed versus starved; †$P < 0.05$ M3 or M15 versus M1; ‡$P < 0.05$ M15 versus M3; N.S., not significant.

	Fed	Starved	Starved/fed ratio	ANOVA
Specific GDP binding (pmol of GDP/mg of protein)				
M1	126 ± 11	176 ± 14*	1.397	Fraction: $P < 0.05$
M3	108 ± 6	147 ± 13*	1.361	Starvation: $P < 0.05$
M15	68.0 ± 4.8†‡	125 ± 11*†	1.838	Interaction: N.S.
Total GDP binding (pmol of GDP/tissue)				
M1	77.3 ± 5.0	116 ± 23	1.501	Fraction: N.S.
M3	68.7 ± 7.7	100 ± 19	1.456	Starvation: $P < 0.05$
M15	55.9 ± 7.1	76.3 ± 6.6	1.365	Interaction: N.S.
Specific UCP content (units/mg of protein)				
M1	203 ± 15	262 ± 14*	1.291	Fraction: $P < 0.05$
M3	170 ± 9	152 ± 7†	0.894	Starvation: $P < 0.05$
M15	244 ± 13†‡	110 ± 7*†‡	0.451	Interaction: $P < 0.05$
Total UCP content (units/tissue)				
M1	123 ± 21	183 ± 40	1.488	Fraction: N.S.
M3	113 ± 33	103 ± 13†	0.912	Starvation: N.S.
M15	195 ± 40	66 ± 4*†	0.338	Interaction: $P < 0.05$
UCP/COX ratio (arbitrary units)				
M1	190 ± 50	162 ± 16	0.853	Fraction: $P < 0.05$
M3	142 ± 27	90 ± 5†	0.634	Starvation: $P < 0.05$
M15	193 ± 22	65 ± 3*†	0.337	Interaction: N.S.

pattern (Table 5). The UCP/COX ratio decreased in fraction M15 and, less markedly, in fraction M3, but remained unchanged in fraction M1. Specific GDP binding was also determined as a measure of functionally active UCP molecules, and was found to be increased after 24 h starvation in all mitochondrial fractions (Table 5). This result contrasts with the decrease in total BAT GDP-binding capacity previously reported in mice subjected to a 36–48 h starvation period [11,22,32].

DISCUSSION

Herron et al. [16] showed by immunocytochemical experiments that, after 24 h of treatment with NA, UCP was completely incorporated into mitochondria of cultured brown adipocytes. On the other hand, it is known that removal of NA from brown-fat-cell cultures is accompanied by a net loss of UCP [17]. Our present results indicate that this loss affects mainly the lightest mitochondria pools (M3 and M15), with comparatively little effect on heavy mitochondria (M1). Remarkably, similar results are obtained in a parallel situation *in vivo* (starvation of mice), which is known to lead to a decrease in NA input into BAT and in the tissue total UCP content [9–12]. However, some differences between the results obtained with the two experimental approaches used are noteworthy. Thus, the decrease in the total UCP level was more marked on removal of NA from brown-fat-cell cultures (31 % decrease) than after starvation for 24 h (18 % decrease). Moreover, this decrease affected both medium (M3) and light (M15) mitochondria of brown-fat-cell cultures, and only light mitochondria (M15) of BAT from starved mice. The simplest way to explain these results is to assume that the decrease in the total cell UCP level is first seen in the lightest mitochondria pool (M15), affecting more mitochondria as its extent grows. In any case, some differences between the results *in vitro* and *in vivo* were to be expected, since it is known that other factors, in addition to the decrease in the NA stimulus, such as changes in thyroid-hormone status, are involved in BAT atrophy in response to starvation [22].

Previous results of our group showed that, in cultured brown adipocytes transiently adrenergically stimulated, the induced UCP was rapidly and specifically degraded after removal of NA, whereas in cells chronically adrenergically stimulated the half-life of UCP was much longer and the degradation was non-specific [17]. These data suggested the existence of two different functional pools of UCP: a rapidly degradable pool of newly synthesized protein and a more stable pool which would be protected against specific degradative systems. However, whether or not these two pools were associated with different mitochondrial subtypes was not determined. The results presented here suggest that when UCP appears in heavy mitochondria it is more stable and is not degraded as quickly as when it appears in light mitochondria. An alternative, non-exclusive, explanation of our results would be that a decrease in the adrenergic stimulation stops further incorporation of UCP into light mitochondria without preventing incorporation of the already existing UCP into heavy (M1) mitochondria, which would then result from mitochondrial differentiation of M3 and M15 precursors.

Decreases in total BAT mitochondrial protein and COX activity, as well as in UCP content and GDP binding, were reported during prolonged starvation (36–48 h) in mice [11,22,32]. From our present results on the effect of a shorter starvation period (24 h), it would seem that BAT atrophy in response to starvation in mice is progressive and is more marked during the second 24 h period of a 48 h fast. Thus, although we already found a diminished total UCP level, no losses of total mitochondrial protein and COX activity could be detected, and

we even found increased GDP binding to BAT mitochondrial subpopulations. Interestingly, it was previously shown that mice maintain their body temperature within the first 24 h of starvation, whereas this temperature falls by 10 °C after 48 h starvation [11]. Taking into account all these data, it would seem that BAT displays a sort of homoeostatic response to short- and medium-term starvation: thus, at least during the first 24 h, the tissue retains its full oxidative capacity, burns fat actively, and is able to dissipate energy as heat in spite of diminished UCP levels, thanks to unmasking of pre-existing UCP molecules, contributing to sustained body temperature. As starvation continues, this mechanism would break down, with a decrease in BAT mitochondrial mass and a fall in body temperature. On the other hand, it should be noted that our mice were younger than those used in previous reports [11,22,32] (4 weeks versus 10–12 weeks) and that involution of BAT, with decreases in the GDP binding capacity and the specific UCP content, is known to occur on aging, at least in the rat [24,33].

Results of our group on the response of BAT to cold showed that during the first 24 h of cold exposure the newly synthesized UCP appeared exclusively in the lightest mitochondrial fractions, M3 and M15, of exposed rats, and thereafter it appeared mainly in the heaviest fraction, M1 [27]. Here, we demonstrate that the UCP incorporated into the lightest mitochondrial fractions is preferentially lost when there is a decrease in noradrenergic stimulation. This conclusion is corroborated by results obtained both *in vitro* and *in vivo*. Taken together, these results strongly suggest that the lightest mitochondrial fractions represent pools of mitochondria with a rapid UCP-turnover response to thermogenic signals, in both whole animals and individual brown adipocytes. In contrast, the heaviest mitochondria should be regarded as more mature mitochondria, with a delayed UCP turnover response to both stimulatory and inhibitory thermogenic signals.

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